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Patch-Clamp Analysis of Membrane Transport in Erythrocytes

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1. Introduction

Among all the models used to study membrane transport, erythrocytes (Red Blood Cells, RBCs) have probably been the most utilised cell type. Radioisotopes fluxes, isosmotic haemolysis, ion content analysis (e.g. flame photometry), or fluorescence techniques have been widely used to characterise the various transporters present in the RBCs membrane. These techniques have allowed the description of several types of transporters such as pumps, specific solute transporters, symporters or antiporters, and even ion channels. However, the physiology of RBCs and their maintenance of homeostasis remains incompletely understood, and electrophysiology has proven, since the first single-channel recording on a human erythrocyte membrane thirty years ago, to be a very useful tool to understand more deeply RBC membrane transport. Why does one use these techniques on a small, non-excitable cell that has long been considered no more than an empty bag of haemoglobin? The diversity of transporters in the RBC membrane, including ion channels, shows that these cells are much more complex than expected. Indeed, ion channels now described in the RBC membrane (from Mammals to other Vertebrates) are implicated in important phenomena and functions throughout the cells lifespan (gas transports, cell volume regulation, differentiation and death). In this chapter, we will describe the main properties of the erythrocyte's membrane transport system, how electrophysiological techniques can be applied, and how they have contributed to the comprehension of erythrocyte physiology with the description of the various ion channels that can be found in RBC membrane.

2. Red blood cell membrane description

2.1 Why one studies red blood cell membrane transport properties?

RBCs are highly specialised cells, present in all vertebrates (except some cold/ice-water fish (Ruud, 1954)). Their main role is the transport of respiratory gases, between tissues and lungs or gills. Encapsulation of the respiratory pigment haemoglobin in a cell in vertebrates has hugely increased the gas transport capacity of blood, and is a key point throughout evolution of the animal kingdom. Erythrocytes are produced in the bone marrow, differentiating from pluripotent cells during erythropoïesis. A human RBC has a lifespan of around 120 days, before being removed from the circulation by macrophages, essentially in the spleen.

RBCs have always occupied a primordial place in the investigations on membrane transport. First of all, even if their major role in respiration processes of vertebrates has been known for long time, deciphering the precise role of the different membrane transporters involved has been a long story. Indeed, membrane transport and especially ion permeability are inseparable from the description of gas transport by erythrocytes. Oxygen diffuses freely across RBC membranes, but its affinity to haemoglobin is highly dependant on cell homeostasis and thus to transport regulation across cell membrane. Moreover, the high carbon dioxide transport capacity of blood is essentially supported by the Jacobs-Stewart cycle between red cells and plasma, relying on the existence of specialised ion transporters in the erythrocyte membrane (Jacobs & Stewart, 1942).

Moreover, the cellular structure and particularly that of mammalian of RBCs, has made them an ideal model for studying membrane transport. Mature mammalian erythrocytes are devoid of intracellular organites, and this means that they consist of a single compartment, simplifying many approaches for transport studies. During the end of cellular differentiation, the nucleus is extruded from the normoblats and engulfed by surrounding macrophages (Yoshida et al., 2005), and the other organelles are removed during the maturation of reticulocytes into erythrocytes, probably mainly via autophagy (Kundu et al., 2008; Mortensen et al., 2010). This makes mature erythrocytes from mammals a very easy-to-use model for plasma membrane transport studies: intracellular constant measurements (ion or metabolite concentrations, pH) and flux experiments are easier than in any other type of cell containing multiple compartments.

Finally, another reason for studying red blood cell membrane transporters is the nature of blood, as a non-fibrous connective tissue : the fact that these cells are naturally in suspension and thus do not need any mechanical, enzymatical or chemical treatment before use in any kind of experiment also makes them easy to handle. Moreover, apart from ethical questions, it is always technically easy to draw blood from animals or humans, and purification of RBCs from blood only requires few centrifugation steps.

Thus, many scientists have used RBCs throughout history to describe the diversity of transporters in the plasma membrane, and to understand their role in the maintenance of homeostasis. Among all these studies, several have revealed essential characteristics of cell membrane permeability. In particular, as early as 1960 work on RBCs allowed Tosteson and Hoffman to complete the description of the "pump and leak" steady-state concept using sheep RBCs (Tosteson & Hoffman, 1960). In 1966, Schatzmann described for the first time an ATP-fuelled Ca^{2+} pump and this discovery was made using human erythrocytes (Schatzmann, 1966). Furthermore, as early as 1981 these cells were among the first using the patch-clamp technique that provided direct electrophysiological evidence for the presence of ionic channels in the plasma membrane (first recordings of a K_{Ca} channel by Owen Hamill) (Hamill, 1981). They were also the cells in which aquaporins were first described and for which Peter Agre won the Nobel Prize (Agre et al., 1993). Nowadays, many studies on the properties of RBC membrane transport are made either in physiological or pathophysiological situations, and the patch clamp technique has become an essential tool in their characterisation and comprehension.

2.2 The basis of red cell membrane permeability

The transport of the respiratory gases within the blood is highly dependent on electrolytes and the acid-base status of RBCs and they are strongly correlated with the permeability

properties of the membrane. Indeed, the erythrocyte membrane is endowed with a variety of membrane transporters, whose role is absolutely vital to maintain cell homeostasis. In this section, we will first present the different types and roles of membrane ion transporters that have been described in human RBCs, as they are the most studied among vertebrates.

The main characteristics of human RBC membrane ion permeability are linked to the unusual composition of these cells. The encapsulation of ~ 5 mmol of impermeable haemoglobin per litre of intracellular water in a cell moving in a plasma environment, that has a much lower protein concentration, creates a huge osmotic pressure. As explicitly formulated in the 'pump-leak' concept (Tosteson & Hoffman, 1960), the risk of colloidosmotic swelling and bursting is prevented by a very low membrane permeability to cations, allowing the pumps Na^+/K^+-ATPase and Ca^{2+}-ATPase to extrude the residual Na^+ and Ca^{2+} leaks at minimal metabolic cost. The red cell Ca^{2+}-ATPase is so powerful that it maintains intracellular concentration below micromolar concentrations (Lew et al., 1982; Schatzmann, 1983). The Na^+/K^+-ATPase maintains gradients for Na^+ and K^+, fuelling the secondary transporters present in the membrane. Indeed, a potassium/chloride cotransporter (KCC, identified as KCC1 (C.M. Pellegrino et al., 1998)), a potassium/sodium/two chloride cotransporter (NKCC) (Haas, 1989) and a sodium/proton (Na^+ /H^+) exchanger (Semplicini et al., 1989) have been described in the RBC membrane.

By contrast, the RBC membrane is characterised by a huge anion permeability that is essentially linked to the respiratory function: a million copies per cell of electroneutral Cl^-/HCO_3^- exchanger (called Band 3) permit 85% of the CO_2 produced in the tissues to be transported in the blood as HCO_3^- ions, *via* the Jacobs/Stewart cycle (Figure 1). This protein was identified in 1972 by Cabantchik and Rothstein (Cabantchik & Rothstein, 1972), even if RBC anion permeability had been studied for long time.

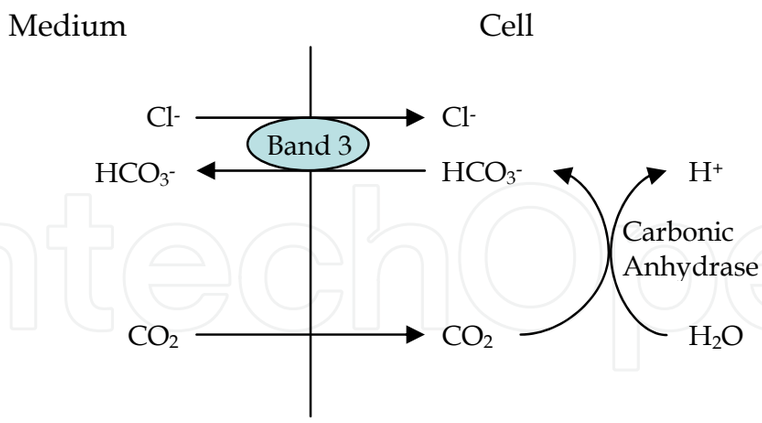

Fig. 1. The Jacobs/Stewart cycle in tissues. In lungs, cycle goes the other way.

It was known for long that RBCs anion permeability could be divided into two components: a large exchange component fundamental for the CO_2-carrying capacity of the blood (Gunn et al., 1973), and a much smaller electrogenic component that normally determines the RBC resting potential (Hunter, 1977; Lassen et al., 1978). This conductive part of chloride permeability ensures a dissipation of chloride gradient across red cell membrane: the membrane potential is clamped at the equilibrium potential for chloride (-12mV) ensuring

that Band 3 never has to fight against a chloride gradient to transport bicarbonate ions across red cell membrane. Before the use of patch clamp, both components were frequently attributed to Band 3 protein activity, the electrogenic part resulting from either slippage in the exchange mechanism (Kaplan et al., 1983; Knauf et al., 1977), or tunneling (Frohlich, 1984; Knauf et al., 1983).

Besides these cotransports, a single conductive pathway had been described in RBCs before the appearance of patch clamp techniques: Gardos had shown in the 50's that an electrogenic, calcium-dependent potassium pathway was present in the human red cell membrane. This has since become known as the Gardos effect, linked to the activity of a calcium-activated potassium channel (Gardos, 1956, 1958).

For a long time, this list of membrane transporters was assumed to be complete: Band 3 mediated the very high anion permeability of red cells (*via* anion exchange and a much smaller electrogenic gating), and the tiny cationic permeability was supported by various powerful pumps and cation transporters carrying out homeostasis maintenance. The emergence of patch clamp studies on red cell membranes (during the 80's and 90's for single channel, 2000's for whole cell) first confirmed the presence of a calcium-activated potassium channel. It was described by single channel recordings by Hamill in 1981 (Hamill, 1981), and identified later as a member of the K_{Ca} channel family (hSK4, now called Gardos channel) (Hoffman et al., 2003). But these patch clamp studies, first using single channel (cell-attached and excised inside-out) and then whole-cell configurations, also brought evidence of a more complex situation than expected. The groups in the fields showed the existence of various anion and cation channels in the red cell membrane from different vertebrate species, and even if their role in physiological situations still remains poorly understood, their implication in several red cell pathologies is unambiguous and considerable.

It has taken much effort to first apply and then adapt patch clamp techniques to these tiny, non excitable cells; especially with the previous models of red cell membrane permeability that did not predicted the presence of various ion channels. Indeed, description of these different channel types in RBCs, using patch clamp techniques, has raised many questions: how do they interfere with the regulation of intracellular homeostasis, cell differentiation or death? But their presence also fits with a new vision of red cells, being much more than an empty bag of haemoglobin, notably regarding the recently discovered role of red cells in vascular tone regulation (Sprague et al., 2007).

3. Technical specificities of red blood cells regarding the patch-clamp technique

The technical aspects described here will focus on human red blood cells, as they are more described and studied than erythrocytes from any other species.

3.1 Red blood cell specificities

3.1.1 Size and deformability

The main problem when attempting to perform patch clamp on RBCs lies in their very small size. The smallest RBCs are encountered in mammals and are enucleated (mean diameter: human 8µm, mammalian 2.1-9.4µm). They are nucleated and slightly bigger in other

vertebrates: amphibian: 16-70µm; Birds: 9.7-15.4µm, fish: 6.5-44.6µm. Nevertheless, deformability of the membrane remains a problem that could impair seal formation. This small size (notably for human RBCs) imposes working with high quality microscopes and objectives (at least X20) and a correct micromanipulation set up.

The pipettes must be really thin, with an average opening below 1µm. The main problem is to avoid entry of the cells into the pipette. RBCs are highly deformable, a property linked to their unique membrane composition and cytoskeleton structure. This allows correct blood circulation *via* all the narrow capillaries that are essential for correct transport of respiratory gases to each cell. In the human circulation, when passing through the spleen, RBCs have to go through tiny slits whose mean size has been recently measured: 1.89µm length and 0.65µm wide (Deplaine et al., 2011). Thus, considerable efforts have been necessary to adapt pipette shape and size for patch clamping RBCs. Their characteristics will be given in section 3.2.

3.1.2 Preparation

Blood is a non-fibrous connective tissue and collection and isolation of RBCs is therefore, a rather easy process. This is a great advantage, meaning that to be patched these cells do not require any mechanic or enzymatic dissociation, and that they do not adhere to solid surface *in vitro*.

Blood must be drawn from human or animal with an anticoagulant (such as heparin or EDTA), and can be stored at 4°C for several days. To isolate RBCs, simple centrifugation steps (usually four successive washings) at a speed of 3000 to 4000 rpm (around 2000g) are needed. RBCs are the densest elements of blood, thus supernatant (plasma) and buffy coat (white blood cells and platelets) can be discarded after each step. RBCs are usually washed with an appropriate saline isosmotic buffered solution.

3.1.3 Cell ion composition

The ion composition of human RBCs is given in Table 1. Plasma concentrations are also given in order to calculate ion gradients occurring in a physiological situation across RBCs membrane.

	Erythrocyte	Plasma
Cations		
Na^+	10 - 20	145
K^+	140	4.5
Ca^{2+}	<0.001	2
Mg^{2+}	1.5 - 2.4	1
Anions		
Cl^-	80	105
HCO_3^-		24
HPO_4^{2-}		4
protein	340g/l	60-80g/l

Table 1. Ion composition of human erythrocytes and plasma (in mM, except for proteins).

3.2 Patch clamping technique on red cells

3.2.1 Remarks on the use of patch clamp on small cells

Erythrocytes are small cells when compared to most commonly studied cells using patch clamp techniques. This induces two different types of problem that had to be considered. The first one was to adapt the shape and size of the pipette, so that a fragment of the membrane could seal to the tip of the pipette without this highly deformable cell entering totally the pipette. The second was that consequent to this adapted shape, electrical issues needed to be considered, especially for the whole cell configuration. Figure 2 describes the electric model for cell-attached configuration and whole-cell configuration.

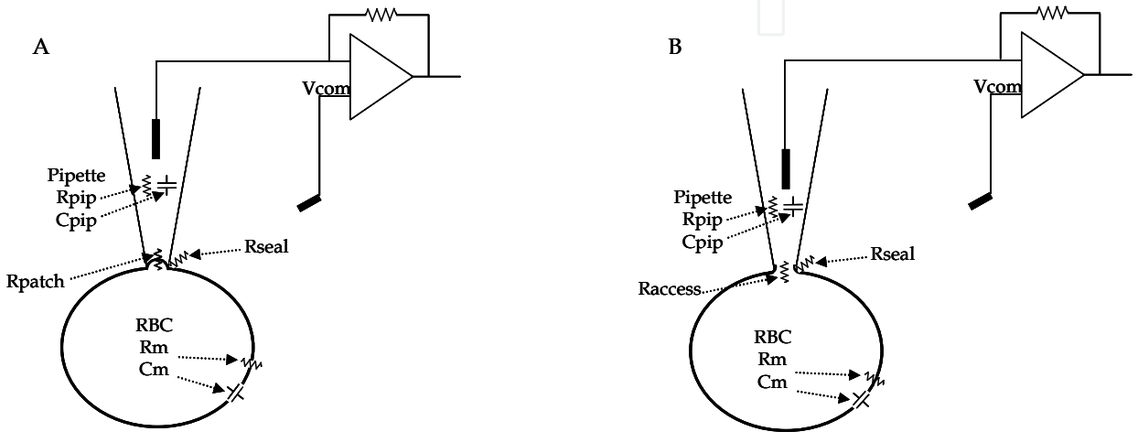

Fig. 2. Electrical model of patch clamp for cell-attached (A) and whole-cell (B) configuration

Single channel studies

When narrowing the tip of the pipette, its resistance R_{pip} is increased. Work on an adapted shape of the pipette for RBCs (especially human RBCs) has led to the use of pipette with an R_{pip} between 10 and 15MΩ. Up to 80% of seal attempts are successful with such pipettes, depending on the solutions used. These values are not a problem for single channel studies, as they remain well below the patch resistance R_{patch} through which single channel currents are recorded, thus these currents can be easily recorded. However, as suggested by Barry and Lynch (Barry & Lynch, 1991), distortion of the potential really applied to the membrane can happen in small cells, in relation with the global membrane resistance of the cell R_m. This resistance (see Figure 2) in small cells can be of the order of magnitude of several gigaOhms, not much lower than R_{patch}. Then variations in the pipette potential can induce changes in the global membrane potential, and this would result in large errors in the estimation of the single channel conductance and of the reversal potential. Barry and Lynch conclude their work (Barry & Lynch, 1991) by the equation (1) revealing the distortion between the apparent and real conductance of the single channel conductance :

$$\gamma_c=\gamma_{app}(1+R_m/R_{patch}) \quad (1)$$

where γ_c and γ_{app} are the real and apparent conductance of the channel, respectively. This clearly highlights the possible distortion of conductance estimation with cells showing a high membrane resistance.

Whole-cell studies

For whole-cell experiments, two resistances are in parallel in the electrical model: global membrane resistance R_m and seal resistance R_{seal} (see Figure 2). Once again, the complexity when using this configuration with small cells is linked to the possible high value of membrane resistance. Seal resistance is barely higher than 100GΩ, and small cell global resistance can reach this order of magnitude. Mammalian T-lymphocytes for example are reported to have a membrane resistance of 100GΩ (Cahalan et al., 1985). Then the contribution of the seal leak to current recorded in this configuration is not negligible: the current measured between the two electrodes can be attributed either to membrane current or seal leak, and the voltage applied to the pipette might be different of the voltage really occurring at the membrane level. This can induce an underestimation of the channel conductance and a discrepancy between real and apparent reversal potentials in cell-attached experiments, and wrong global conductance and shift in I/V curves in whole-cell experiments.

Do these limitations apply to RBCs?

From suspension experiments, membrane resistance R_m of human RBCs was estimated in the range $1.10^6 \Omega.cm^2$, with chloride resistance R_{Cl} ranging between 10^5 and 10^6 $\Omega.cm^2$ (Hoffman et al., 1980). Given a mean surface of $135\mu m^2$, the whole-cell resistance can be estimated at 10 to 40 GOhms. This is in range of the seal values obtained on RBCs when using the patch clamp techniques. This explains why whole-cell experiments were not used on red blood cells: the conductance was estimated to be too low to discriminate properly between channel activity and seal leakage. However the membrane resistance can be considerably lowered when the ion channels present in the red cell membrane are activated: anion conductance rises to several nS in PKA-activated, oxidized or malaria-infected RBCs, and cation conductance can be increased when cation channels are activated (with low external chloride concentrations for example). Thus, for whole cell experiments, R_m remains much lower than R_{seal} and the current and voltage do not suffer high distortion.

Another parameter to take into account is the pipette access resistance R_{access}. Since the pipette tip must be rather narrow when patching RBCs, precautions must be taken. R_{access} value is determined by pipette geometry, solution composition and possibly by the presence of cell debris generated by membrane rupture. The use of pipette immediately after pulling, their adapted shape, with rapidly tapering geometry, and the filtering of all pipette solutions with 0.2µm filters can maintain low R_{access} values.

3.2.2 Electrodes, pipettes and seal

In our group, we use Ag/AgCl electrodes. Ag wires are regularly anodised to maintain uniform oxidized coating. The reference electrode is connected to the bath solution *via* an Agar/KCl 3M bridge. When perfusion of bath solution is performed, it modifies junction potentials. Liquid junction potentials must be corrected *a posteriori*. For that purpose, the JPCalc software developed by Peter Barry is very convenient (Barry, 1994).

Pipettes must be pulled by good pullers and have a tip diameter well below 1µm. We use thin borosilicate pipette with filament (this helps liquid filling) (GC150TF-10, Clark Electromedical Instruments), and pull them with a horizontal DMZ puller (Zeitz instruments, Germany). The pulling is concluded by automatic heat-polishing step ensuring

highly reproducible geometries and electrical properties. The pipette resistance in physiological saline solutions is usually around 10-15MΩ. The pipette capacitance can be measured and compensated, it is usually around 3pF.

As mentioned previously, RBCs are non-adhesive cells; thus pipettes can approach the bottom of the plate, using a 10X and then a 40X objective (Figure 3a-b). When the pipette is in contact with a RBC a small, calibrated depression can be applied (around 20mbars) and the cell can be lifted above the supporting plate (Figure 3c). The seal formation is helped by this negative pressure, and by the presence in the pipette solution of divalent cations (Mg^{2+} and/or Ca^{2+}). A pulse of 10mV is imposed on the pipette to monitor the resistance of the contact between membrane and pipette (seal resistance), from a holding potential around -30mV (figure 3d). When the seal value is higher than 10GΩ, the system is ready for cell-attached recordings.

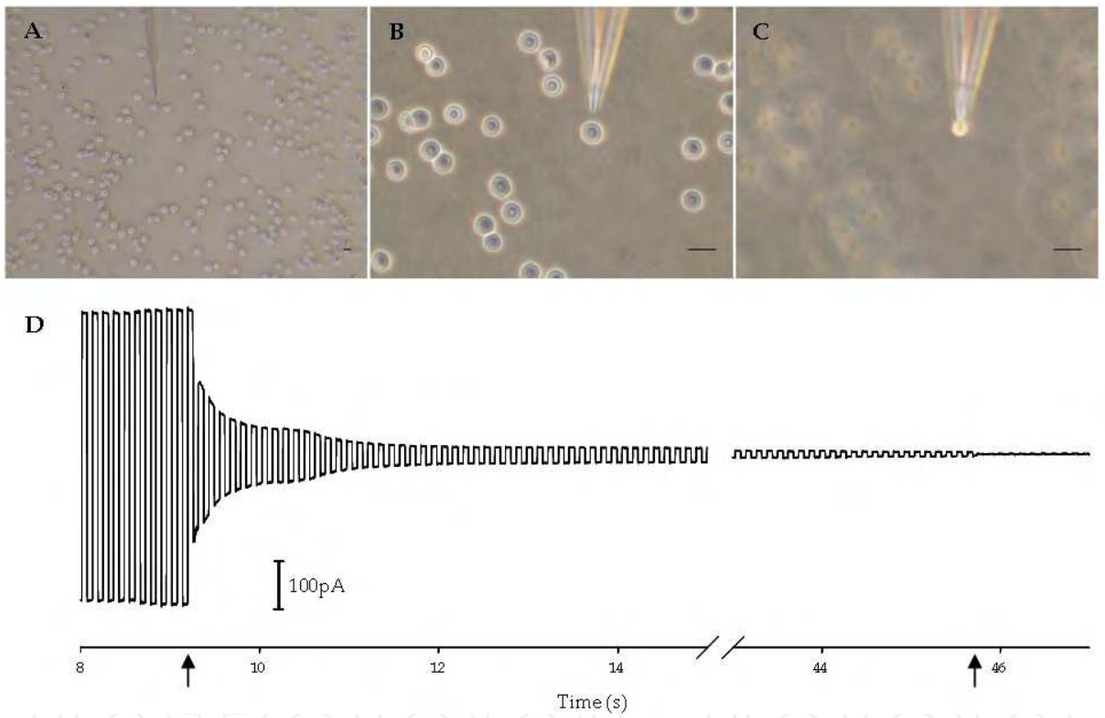

Fig. 3. Human RBC patch clamp - approach, seal and electric monitoring of seal formation. A, pipette approach with 10X objective. B, pipette approach with 40x objective. C, Patched RBC. D, monitoring of cell/pipette contact resistance (seal formation). Cell is touched by the pipette after 9s (arrow) with calibrated depression, and seal (around 10GΩ) is complete after 45s (arrow). Scale bar for A, B and C : 10µm.

3.2.3 Single channel recordings

The ion channels present in the RBC membrane can have a small unitary conductance, thus the most important task is to track and eliminate noise. Efficient Faraday cages and link of all metallic elements (microscope, anti-vibration table, and micromanipulator) to the ground using low resistance cables are of primary importance. Noise is also dependent on filtering settings. In our studies, recordings are filtered with a low pass 3 kHz filter. This allows

correct transition detection of events up to 300µs, and elimination of noise faster events. When needed, these records can be refiltered digitally during analysis, with lower low-pass cut-off. Before digitalisation, signal is displayed on an oscilloscope to monitor analogic signal live and continuously. We use a 1401 or micro 1401 acquisition interface (Cambridge Electronic Design, Cambridge, UK) for analogic/digital conversion. Sampling rate is set at 10 kHz, above the Nyquist–Shannon sampling theorem value. Recordings for each voltage should last at least 1min, to obtain a sufficient number of events that will allow correct kinetic analysis (dwell time, open probability, burst duration). From cell-attached configuration, excised inside-out configuration of the patch clamp can be obtained by exposing very briefly the patched cell to air (this will rupture the membrane around the pipette tip). Then perfusion can modify the intracellular side solution and help characterising the channel activity.

3.2.4 Whole-cell recordings

After obtaining seal, records can be made for single channel studies. For whole-cell studies, the fragment of membrane inside the tip of the pipette must be ruptured. This is achieved via imposition in the pipette of a brief electrical pulse (200ms, 500mV). This rarely provokes damages to the seal. Successful whole-cell configuration can be checked via the sudden appearance of membrane capacitance transient currents. Mammalian erythrocyte capacitance is estimated at 0.8 $\mu F.cm^{-2}$ (Fettiplace et al., 1971), this gives a membrane capacitance around 1-1.3 pF for red blood cells. A measure of membrane capacitance for human RBC gives a value of 1pF (Rodighiero et al., 2004), and this can be compensated on the amplifier. Seal leak contribution can be estimated. If seal resistance is around 10GΩ, current leaking at +100mV can be calculated at 10pA. We usually have recordings showing current values well above 300 to 1000pA; then leak always remains below 1-3% of total current.

The nystatin-perforated patch clamp has also been used on human RBCs, especially to study cation channel activity. This technique avoids the dialysis of important substances from the RBCs cytoplasm. In these studies, nystatin was used at a final concentration of 150µg/mL in the pipette (Rodighiero et al., 2004; Vandorpe et al., 2010).

4. The contributions of patch clamp techniques in describing human red blood cell membrane properties

In this part of the chapter, we will list the various ion channels that have been described or suggested in the human RBC membrane, and list their main properties as well as recording conditions. Their possible physiological role will be evoked, before a description of their implication in various pathological situations.

4.1 Cation channels

4.1.1 Gardos channel

As mentioned previously, the Gardos channel was the first channel described in RBCs membrane. The pioneering experiments were performed by Georges Gardos who showed that metabolic poisoning of human RBCs was able to elicit K^+ leak via an ion channel depending on Ca^{2+} (Gardos, 1956, 1958). The phenomenon was termed Gardos effect; and later on this was particularly studied, since it was shown that the Gardos channel plays a

role in cell dehydration during sickling at low oxygen tension in sickle cell disease (see §4.3.1). Then in the early years of patch clamp in 1981, RBCs were used to set up the technique, and Owen Hamill demonstrated using cell-attached recordings that the Gardos effect was truly supported by the activity of a Ca^{2+}-activated K^+ channel (Hamill, 1981). The channel remained known as the Gardos channel. It was later identified as the SK4 channel, belonging to the Small Conductance Calcium-Activated Potassium Channel family (SK channels). It is encoded by the gene kcnn4 (Hoffman et al., 2003). In an attempt to reconcile fluxes and electrophysiological data, Grygorczick and Schwarz estimated the number of channels per cell to be between 10 (Grygorczyk & Schwarz, 1983) and 55 (Grygorczyk et al., 1984). However, using inside-out vesicles from RBCs and $^{86}Rb^+$ fluxes, Alvarez and Garcia-Sancho estimated this number to be around 150 (Alvarez & Garcia-Sancho, 1987). Today this value seems the closer to reality. Until now, the Gardos channel remains the best characterised channel in human RBC membrane.

Examples of Gardos channel activity are given in Figure 4, showing cell-attached recordings. It was recently shown that the deformation of the membrane during seal formation was able to induce transient activity of the Gardos channel, mainly due to the entry of Ca^{2+} via a finite Ca^{2+} permeability (Dyrda et al., 2010). This induces a quick loss of cellular potassium via Gardos channel activation in all the membrane. Then when using KCl in the pipette solution, the current remains inwardly directed.

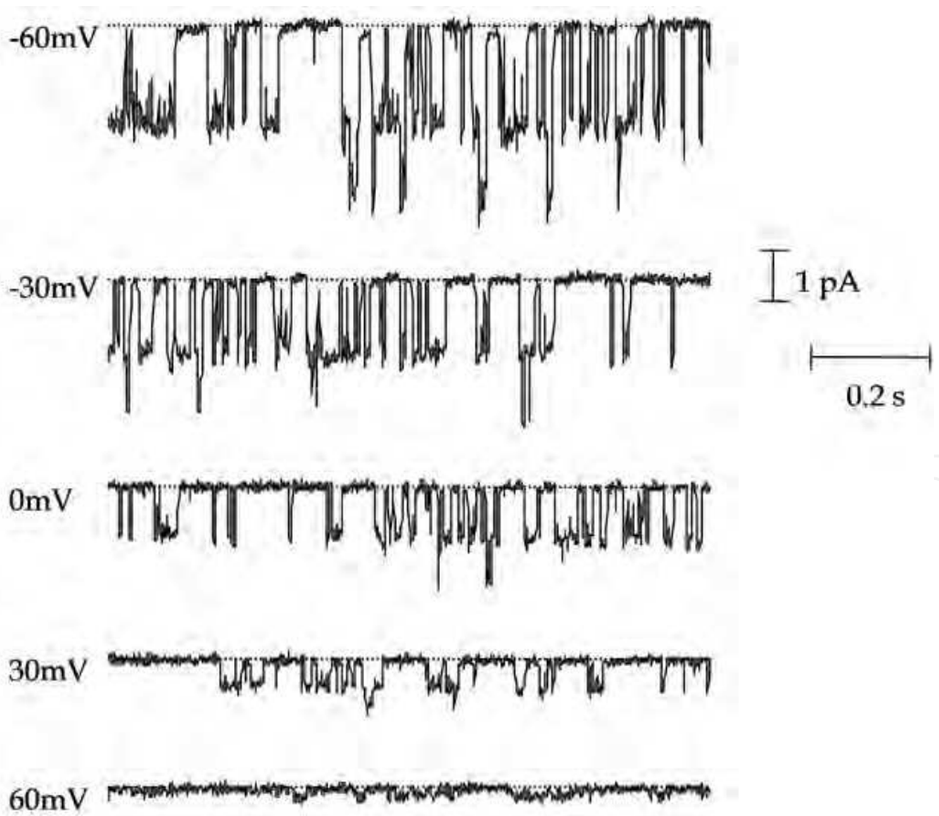

Fig. 4. Example of Gardos channel activity
Bath solution (mM): 115 NaCl, 5 KCl, 10 $MgCl_2$, 1.4 $CaCl_2$, 10 Hepes, 10 glucose. Pipette solution (mM): 120 KCl, 10 $MgCl_2$, 1.4 $CaCl_2$, 10 Hepes, 10 glucose. Voltages indicated refer to –Vp.

When recorded with a K^+ concentration of 100mM in the bath and pipette, the Gardos channel displays a conductance of 18pS (Hamill, 1981), or 25pS with 140mM K^+ (Grygorczyk et al., 1984). The channel shows slight inward rectification at high voltages (Grygorczyk et al., 1984). The open probability does not appear to be voltage-dependant. The channel has a clear selectivity for K^+, with pK^+/pNa^+ ~ 15 (Grygorczyk & Schwarz, 1983). However, Christophersen gave a more precise permeability sequence for the Gardos channel, from bi-ionic reversal potential (Christophersen, 1991): $K^+ \geq Rb^+ > NH_4^+ >> Li^+$; Na^+; Cs^+. The channel shows a susceptibility to temperature, with increased conductance and reduced open probability with increasing temperature (Grygorczyk, 1987).

Ca^{2+} at the intracellular face of the channel is necessary for channel activation. Open probability raises from 0.1 to 0.9 with an increase in free $[Ca^{2+}]_i$ from 500nM to 60µM. It is admitted that 2-3 µM of free $[Ca^{2+}]_i$ is required to activate the channel (Yingst & Hoffman, 1984). Calcium acts via binding to calmodulin, which is constitutively associated with the Gardos channel (Del Carlo et al., 2002). Protein kinase A induces a dramatic increase in Gardos channel activity, an effect that might be linked to enhancement of Ca^{2+} sensitivity (M. Pellegrino & Pellegrini, 1998). Lead is also a known activator of the Gardos channel, and it was shown that Pb^{2+} ions act independently and on the same site as Ca^{2+} ions (Shields et al., 1985). NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) is also a powerful agonist that decreases $K_{1/2}$ (Ca^{2+}) for IK and SK channels (Strobaek et al., 2004), and can be used to activate Gardos channel at subphysiological extracellular Ca^{2+} concentrations (Baunbaek & Bennekou, 2008). Extracellularly applied clotrimazole (Brugnara et al., 1993b) and charybdotoxin (Brugnara et al., 1995; Brugnara et al., 1993a) inhibit Gardos channels with IC50s of 50 nM and 5 nM, respectively. On the intracellular side, when the inside-out configuration is used, Ba^{2+} and tetraethylammonium exert a voltage-dependent channel inhibition by binding to the cytoplasmic domains of the channel with Kds of 150nM and 20mM, respectively (Dunn, 1998). In addition, the channel activity needs extracellular potassium, and incubation of RBCs ghost in a K^+-free solution irreversibly inhibits the activation of the channel by Ca^{2+} (Grygorczyk et al., 1984).

The resting free $[Ca^{2+}]_i$ in an unstimulated cell is about 100nM, consequently Gardos channel activation in a physiological situation seems rare. Thus, the physiological role of the channel still remains unclear. However, since deformation tends to activate the Gardos channel (at least indirectly) (Dyrda et al., 2010), the presence of the Gardos channel could make sense during the normal aging of RBCs. Density of RBCs is known to increase during the 120 days of their lifespan, and the channel has been involved in this process, with a decrease in maximal activity of the channel with cell age (Tiffert et al., 2007). A recent study using Gardos channel knock-out mice also showed modulation of various RBCs parameters. Mouse Gardos-deficient RBCs are mildly macrocytic, their susceptibility to osmotic lysis is increased and their filterability is impaired (Grgic et al., 2009), suggesting a role for Gardos channel in RBCs volume maintenance. Besides, the Gardos channel is also involved in various pathophysiological situations, notably in sickle cell anaemia where part of the cell dehydration leading to sickling is *via* the Gardos channel (Lew et al., 2002).

4.1.2 Non-selective cation channel

A voltage-activated cation channel in the human RBC membrane was originally proposed by Halperin (Halperin et al., 1989), and it was later described electrophysiologically using

single channel recordings (Christophersen & Bennekou, 1991). This channel was further described by Bennekou (Bennekou, 1993), and by Kaestner (Kaestner et al., 1999). The channel is coupled to an acetylcholine receptor of nicotinic type (Bennekou, 1993), and can be activated by prostaglandin E2 (Kaestner & Bernhardt, 2002) and clotrimazole and its analogues (Barksmann et al., 2004). The channel shows a conductance around 20pS (Christophersen & Bennekou, 1991), and is permeant to mono- and di-valent cations, including Ca^{2+}, Ba^{2+} and Mg^{2+}. It shows a hysteresis voltage dependence (Kaestner et al., 2000), that was shown using patch clamp as well as cell suspension potential measurements (Bennekou et al., 2004a). Indeed, the half-maximal voltage of activation curve is 25mV higher than for the deactivation curve. The voltage-activated non-selective cation channel was also described using whole-cell and nystatin-perforated patch-clamp recordings, with a half-maximal conductance reached at 42mV (Rodighiero et al., 2004). It is inhibited by Ruthenium Red, N-ethylmaleimide (NEM), La^{3+}, or iodoacetamide (Bennekou et al., 2004b).

During the last decade the group of Florian Lang has also described a voltage-independent cation channel in the human RBC membrane. This channel can be activated by oxidative stress (Duranton et al., 2002), hyperosmotic shrinkage (Huber et al., 2001), and replacement of extracellular chloride by gluconate, NO_3^-, Br^- or SCN^- (Duranton et al., 2002; K.S. Lang et al., 2003). Monovalent cations are barely discriminated by this channel, with a selectivity $Cs^+ > K^+ > Na^+ = Li^+ >>> NMDG^+$ (Duranton et al., 2002); the channel is also permeable to Ca^{2+}. This voltage-independent channel is inhibited by amiloride, gadolinium or EIPA (ethylisopropylamiloride) (Duranton et al., 2002).

One cation channel seems to be identified as the Transient Receptor Potential Cation channel 6 (TRPC6), which fits with properties of the non-selective voltage-dependent cation channel. Expression has been detected in erythroid progenitor cells, and the protein has been detected in mature erythrocyte membrane by western blot analysis (Foller et al., 2008b).

The non-selective cation channels described so far in RBCs membrane are permeant to Ca^{2+}, leading to the hypothesis that the conductive pathway for Ca^{2+} entry is such a route. Nevertheless, a few studies have given evidence that true Ca^{2+} channels might also be present in the RBCs membrane. Even if the data regarding the exact nature of the Ca^{2+} pathway are confusing, the presence of both L-type and R-type Ca^{2+} channels subunits in RBCs membrane has been detected using western blots and tracers fluxes with adapted inhibitors (Romero et al., 2006). The group of Pedro Romero has also shown that the channel types appear to be different according to the RBC age. The pharmacology experiments suggest activating effects of vanadate (Romero & Romero, 2003) and caffeine (Cordero & Romero, 2002) on these Ca^{2+} channels. However, until now these channels have never been characterised electrophysiologically, but only with tracer fluxes using $^{45}Ca^{2+}$. The work of Pinet *et al* (Pinet et al., 2002) also suggests that the Ca^{2+} pump in human RBCs may behave as a Ca^{2+} channel sharing many similarities with the B-type Ca^{2+} channel. It can be seen when adapted voltage protocols are used on RBCs or when the Ca^{2+} pump is inserted into liposomes.

Thus, it seems that at least two distinct cationic pathways coexist in the human RBC membrane. Their physiological roles are probably mainly linked to regulation of free $[Ca^{2+}]_i$. It is known that prostaglandin E2 increases free $[Ca^{2+}]_i$ leading to Gardos channel activation

(Li et al., 1996), and decreases in erythrocyte deformability and filterability with a maximal effect at 0.1 nM (Allen & Rasmussen, 1971). Beyond Gardos channel activation and cell shrinkage, an increase in free $[Ca^{2+}]_i$ could also lead to scramblase activation and phosphatidylserine exposure on the outer membrane leaflet. Cation channels are thus implicated in the cascade of events leading to RBCs death, a phenomenon called eryptosis (as an erythrocytic-specific apoptosis) and described by the group of Florian Lang (Foller et al., 2008a; F. Lang et al., 2006; K.S. Lang et al., 2005).

4.2 Anion channels

As described in the first part of this chapter, conductive anion permeability of the RBC membrane has long been exclusively attributed to band 3 via slippage, or tunneling mechanisms. Nevertheless, various anion channel activities have been described essentially in the last decade, giving unambiguous evidence of the presence of anion channel in the human RBC membrane. Knowledge has come partly from studies on *Plasmodium*-infected RBCs, showing spontaneous anion channel activity. It is now known that these channels are endogenous proteins, upregulated in parasitized cells. Nonetheless, anion channel inhibitors are generally poorly specific, and in cells lacking expression machinery, and where membrane majoritary proteins complicate proteomics studies, precise identification of these anion channels has been and still remains difficult. So far two functional anion channels have been identified in the human RBC membrane, plus the CFTR channel that probably acts more as a regulator.

4.2.1 Cystic Fibrosis Transmembrane conductance Regulator (CFTR)

The presence of CFTR protein in the human RBC membrane has long been debated. CFTR transcripts in human RBCs progenitors and CFTR protein were not found by Hoffman et al. (Hoffman et al., 2004). On the contrary, other groups using various techniques suggested the presence of this channel in the RBC membrane. Indeed, CFTR activity seems necessary for deformation-induced ATP release and the protein was detected using western blots (Abraham et al., 2001; Sprague et al., 1998). Moreover, using atomic force microscopy combined with quantum-dot-labelled anti-CFTR antibodies, Lange et al. could estimate the number of CFTR per cell at around 700 and 170 in RBCs from healthy donors and Cystic Fibrosis patients (homozygous ΔF508 mutation), respectively (Lange et al., 2006).

However, no recording of CFTR activity in the RBC membrane at the single channel level has ever been given. Comparisons between RBCs from healthy donors and CF patients using whole-cell configuration allowed the description of a tiny current attributed to CFTR activity; but the role of CFTR remains unclear. Indeed, its regulatory properties seem more important. Plasmolysis, or *Plasmodium*-infection induced a chloride current (different from CFTR activity) in normal RBCs, but not in RBCs from CF patients (Verloo et al., 2004). This was confirmed at the single channel level in non-infected human RBCs, with behaviour similar to a small anionic channels (around 10pS) in these two types of cells: its gating and kinetics were different, but the properties did not correspond to CFTR activity (Decherf et al., 2007).

This seems to confirm the presence of a CFTR protein in the human RBC membrane acting rather as a regulator of other channels/transporters.

4.2.2 Small conductance chloride channel / ClC-2

A small anion channel has been described in the human RBC membrane, and was designed as a ClC-2 channel. Western-blots showed its presence in the human RBC membrane, in keeping with swell-activation and Zn^{2+} inhibition of a whole-cell chloride conductance (Huber et al., 2004; Shumilina & Huber, 2011).

We also described a small conductance chloride channel, spontaneously active using cell-attached configuration in malaria-infected human RBCs, showing a conductance of 4-5pS (Bouyer et al., 2006). Relying on the number of apparitions of active channel, we estimated the density of this channel to be approximately 60-80 copies per cell. In a subsequent study, we showed that this channel corresponds to the ClC-2 channel already described, thus being an endogenous channel activated upon infection (Bouyer et al., 2007). Using the whole-cell configuration it was shown that this channel is activated by oxidation using 1mM *tert*-butylhydroperoxide (t-BHP) (Huber et al., 2004; Huber et al., 2002). Zinc shows an inhibitory effect, with an IC50 around 100µM, whereas NPPB or furosemide are relatively ineffective (Shumilina & Huber, 2011).

This channel might contribute to the basal anionic conductance of human RBCs, clamping the cell potential to the chloride equilibrium potential. Since ClC-2 channels are activated by cell swelling in many cell types (Roman et al., 2001; Strange et al., 1996) including RBCs (Huber et al., 2004), a role for this channel in cell volume regulation seems more than probable.

4.2.3 Voltage-dependent anion channel / peripheral benzodiazepine receptor

Two recent works, using both cell-attached and whole-cell configurations have provided evidence for the presence of another type of anion channel in human RBC membrane. The first study showed that a maxi-anion channel is present in the human RBC membrane (Glogowska et al., 2010). This channel shows multiple conductance substates that are dependent on the presence of serum in the bath solution. Activity was classified in various patterns, with a basal level in the absence of serum showing a conductance of 8-12pS. Multiple substates were described in the presence of serum in the bath, with a maximal conductance of 600pS and a more stable conductance of 300pS. Figure 5 gives examples of this maxi-anion channel activity. The channel showed a preference for SCN^- ions, since some of these substates could be seen in serum-free conditions when cells were bathed in solutions where 10mM of Cl^- ions was replaced by SCN^- ions.

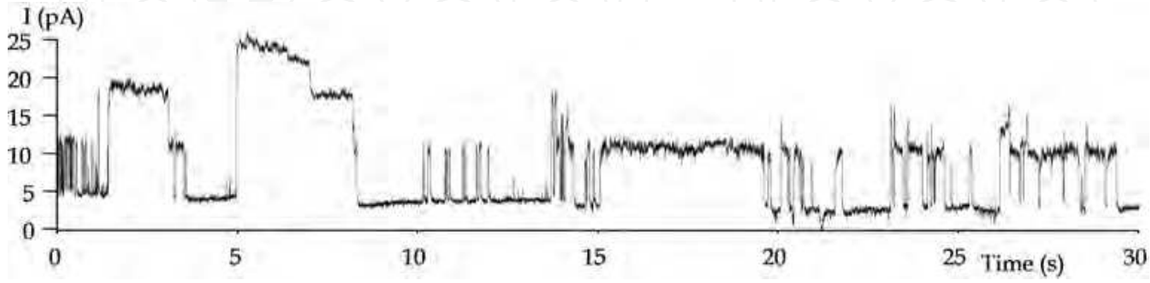

Fig. 5. Example of VDAC recordings with multiple conductance substates.
Bath solution (mM): 115 NaCl, 5 KCl, 10 $MgCl_2$, 1.4 $CaCl_2$, 10 Hepes, 10 glucose plus 0.5% human serum
Pipette solution: 115 NaCl, 5 KCl, 10 $MgCl_2$, 1.4 $CaCl_2$, 10 Hepes, 10 glucose
-Vp=50mV

The identity of this channel remained undetermined until a second study by our group showed that a Voltage Dependent Anion Channel (VDAC) was present in the human RBC membrane (Bouyer et al., 2011a). VDAC are components of the Peripheral Benzodiazepine Receptor (PBR), composed of at least three components: VDAC, an Adenine Nucleotide Transporter (ANT) and a translocator protein (TSPO) that probably modulates VDAC activity (Veenman et al., 2008). Presence of binding sites for specific ligands of this receptor in the RBC membrane had already been suggested (Canat et al., 1993; Olson et al., 1988), and in this study evidence for presence of transcripts in differentiating erythrocytes and of proteins in mature erythrocytes was given (Bouyer et al., 2011a).

VDAC conductance and selectivity are voltage-dependent and shows multiple substates with permeability for small and large anions (SCN^-, Cl^-, glutamate, ATP …), it also displays low conductance substates with cation permeability (Na^+, K^+, Ca^{2+}) (Bathori et al., 2006; Benz et al., 1990; Gincel et al., 2000; Rostovtseva & Colombini, 1997). The channel activity can be activated, or modulated by multiple ways including phosphorylation by PKA, oxidation, Ca^{2+} ions, or serum components (Banerjee & Ghosh, 2004; Bera et al., 1995; Madesh & Hajnoczky, 2001; Shoshan-Barmatz et al., 2010). The channel is sensitive to DIDS, La^{3+} or ruthenium red (Shoshan-Barmatz & Gincel, 2003). In the PBR complex, three specific ligands are generally used as modulators of ion transport activity: PK11195, Ro5-4864 and diazepam. The TSPO component is considered to be primarily responsible for binding to PK 11195, while Ro5-4864 and other benzodiazepines may bind to all components of the PBR complex (Le Fur et al., 1983; McEnery et al., 1992).

The presence of such proteins in the RBC membrane raises many questions regarding their possible physiological role, but according to the properties of its components we can predict a major role in membrane transport, volume and redox status regulation, as well as cell differentiation and senescence. The multiple behaviours of VDAC allow us to propose a unifying hypothesis where VDAC could explain parts of both anionic and cationic channel activity already described in human RBCs, as suggested in a recent review (Thomas et al., 2011). VDAC could then be a common pathway for Ca^{2+} entry (leading to Gardos channel modulation), Cl^- equilibrium regulation or ATP release.

4.3 Implication of RBC ion channels in pathophysiological situations

Although the physiological role of ion channels in the RBC membrane is still under investigation, their implication in various pathologies has been known for decades. In particular, cation channel activity is a key factor of sickle cell disease pathology (Lew & Bookchin, 2005), as is anion channel activity in *Plasmodium*-infected human RBCs (Ginsburg et al., 1983; Kirk et al., 1994). Implication of the channels in senescence of RBCs will also be evoked.

4.3.1 Sickle cell disease

In the sickle cell disease polymerisation of HbS haemoglobin under deoxygenated conditions leads to cell dehydration via efflux of potassium, chloride and osmotically obliged water. Pathways for these effluxes are the Gardos channel and the potassium-chloride cotransporter KCC. Both are activated during sickling consequent to the entry of calcium in the red cell, consecutive of HbS polymerisation, via an abnormal cation

permeability pathway termed P_{sickle}, known and described from a functional point of view, but never characterised at the molecular level. P_{sickle} is a poorly selective permeability pathway for small inorganic mono- and divalent cations (Joiner et al., 1993). Ion movements are non-saturable, voltage-dependent and not obligatory coupled, characteristics of a conductive pathway (Joiner, 1993). However, studies run in two laboratories in Oxford and Cambridge have also reported that under certain circumstances, P_{sickle} might also be permeable to non-electrolytes (Ellory et al., 2007; Ellory et al., 2008), including sugars (notably sucrose or lactose) and other molecules such as taurine or glutamine. This pathway has been characterised mainly using fluxes or haemolysis experiments, yet patch-clamp has also been recently used. Whole-cell cationic conductance can be induced by deoxygenation of HbSS cells. The channel shows equal permeability for Na^+, K^+ or Ca^{2+}, and is inhibited by DIDS (100µM), Zn^{2+} (100µM) and Gd^{3+} (2µM) (Browning et al., 2007). Nystatin-perforated whole-cell recordings and single channel recordings gave a unitary conductance of 27pS, and a sensitivity to dipyridamole (100µM) and GsMTx (*Grammastola spatulata* mechanotoxin IV) (1µM) (Vandorpe et al., 2010).

P_{sickle} is an obvious target of primary interest in the pharmacology of sickle cell disease, because of its initial role in the cascade of events leading to cell dehydration. Future research should focus on its identification. The Gardos channel is also a molecular target in the pharmacology of sickle cell disease, and research is in progress using of clotrimazole and various analogues (Brugnara, 2003). Finally, RBC dehydration in sickle cell disease is also mediated by anion channels and by loss of Cl^- accompanying K^+ loss via the Gardos channel. Thus, these anion channels are another target of interest, an hypothesis successfully tested in seminal work, where specific RBCs anion conductance inhibitors were used to prevent cell dehydration either *in vitro* or *in vivo* on the SAD mouse model for sickle cell disease (Bennekou, 1999; Bennekou et al., 2001; Bennekou et al., 2000).

4.3.2 Malaria and *Plasmodium*-infection

Another pathophysiological situation, where anion channels display a critical role is malaria, caused by infection by parasites of the genus *Plasmodium*. The malaria parasite invades and multiplies within RBCs in about 48h. To accomplish this cycle and ensure the supply of nutrients and the release of waste products, it relies on large, poorly selective anion channels in the host RBC membrane. Because of a dramatic gap in the original knowledge on RBC native anion channel, this transport properties have been termed New Permeability Pathways (NPPs) (Kirk, 2001) and their molecular nature has long been debated. This pathway was characterised using fluxes and haemolysis experiments during the 80's and 90's. Patch-clamp techniques were first applied to infected RBCs by the group of Sanjay Desai who demonstrated an inwardly rectified anion current in infected RBCs (Figure 6) (Desai et al., 2000). Electrophysiological description of the spontaneously active ion channels in infected RBCs membrane has been highly controversial, owing to the multiplicity of experimental conditions used by the different groups in the field. Indeed, a negative holding potential imposed between ramps in whole-cell experiments inactivates inward currents, and the presence of serum in the bath solution activates outward and inward current in a different manner (see Figure 6) (Staines et al., 2003). It was also shown that supraphysiological ionic concentrations used in bath and pipette solutions modify anion channel activity: saturation of conductance and inhibition by lower open probability appeared beyond 0.6M of Cl^- in solutions (Bouyer et al., 2007).

Several studies have since showed that this channel activity was supported by endogenous channels activated upon infection and that the channels were sensitive to PKA phosphorylation, oxidation or serum presence (Bouyer et al., 2007; Bouyer et al., 2011b; Egee et al., 2002; Huber et al., 2005; Huber et al., 2002; Merckx et al., 2008; Staines et al., 2003). Examples of whole-cell currents are given in Figure 6. One channel involved is the ClC-2 channel, but its activation is rather a side effect, since growth of *P. berghei* is not affected in ClC-2 knock-out mice (Huber et al., 2004).

We recently showed that the main component of NPPs was the PBR complex including the VDAC channel, since the specific PBR ligands could prevent both parasite growth *in vitro*, NPP-mediated sorbitol permeability and whole cell anion currents (Figure 6) of infected cells (Bouyer et al., 2011a).

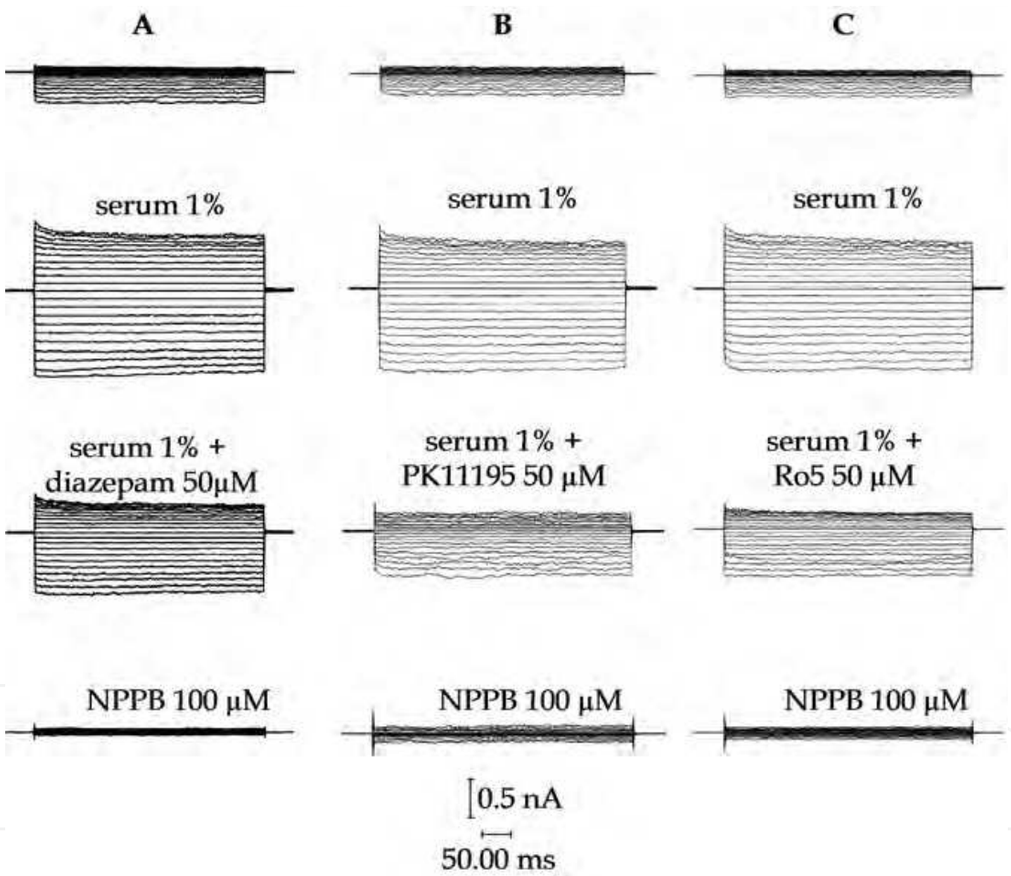

Fig. 6. Anion channel activity in *Plasmodium falciparum*-infected human RBCs
3 cells A, B and C were studied using the whole-cell configuration, and serial perfusion were performed showing the typical serum effect and the inhibitory effect of PBR ligands and NPPB, as described in (Bouyer et al., 2011a). Stimulation was made with 500ms ramps between +100mV and -100mV, with -10mV increments

The identification of PBR/VDAC activity in the *Plasmodium*-infected RBC membrane is an important step in the description of the pathophysiology of malaria. This suggests that the pharmacopoeia of benzodiazepine, as well as benzodiazepine scaffolds for the production of

new inhibitors could become a novel strategic approach for future antimalarial chemotherapies.

The discrepancies between results obtained by patch-clamp studies on infected RBCs in the last decade proved how important the specific experimental conditions are. Indeed, presence of various substances in the bath, holding potential, ionic concentrations, or duration of recordings are able to alter ion channel activity and extrapolation of results to physiological situation is then difficult (Staines et al., 2007). Thus, extra precaution should be taken when designing experiments and analysing results and detailed methodology should be clearly stated in communications.

4.3.3 Senescence

During their circulatory life, RBCs tend to become progressively denser. This correlates with a decline in Ca^{2+} pump activity that leads to KCl loss (via Gardos and anion channels) overcompensated by NaCl gain (Lew et al., 2007). Activity of a non-selective cation channel has been linked to this phenomenon that tends to dissipate Na^+ and K^+ gradients late in the lifespan of RBCs.

A cascade of events leading to programmed cell death of RBCs has been described by the group of Florian Lang. This follows the same path: an external signal (oxidative stress, for example) triggers a rise in intracellular Ca^{2+}, probably by activation of a non-selective cation channel by prostaglandin E2. This provokes cell shrinkage, scramblase and calpain activation resulting in phosphatidylserine exposure and degradation of the cytoskeleton (reviewed in (Foller et al., 2008a; F. Lang et al., 2004; F. Lang et al., 2006)).

Thus, the non-selective cation channel Gardos channel together with anion channels play a central role in RBCs aging process and cell death.

5. Comparative physiology: Use of patch-clamp in a evolutionary approach on vertebrates red cells

Comparative physiology of red blood cells membrane have been evident for many years regarding respiratory function and many studies highlighted the role of ion transporters in resting and challenging situations. Although, the use of the patch-clamp technique on models other than mammals are relatively limited. Nonetheless, major groups have been studied and Table 2 summarizes current knowledge on vertebrates' erythrocytes ion channels.

As stated in the table below, most of the studies performed so far were conducted in the framework of cell volume regulation. Volume regulation is of importance to cells exposed to anisotonic extracellular media and to cells where solute transport could change intracellular osmolality. Exposure of vertebrate cells to a hypotonic solution results in an initial increase in cell volume due to the relatively rapid influx of water. During continuous hypotonic stress increases in cell volume are followed by a slower, spontaneous recovery towards the pre-shock level, a process known as regulatory volume decrease (RVD). This recovery is accomplished by selectively increasing the permeability of the plasma membrane during cell swelling to allow for efflux of specific intracellular osmolytes, thereby generating a driving force for water efflux. Most vertebrate cells lose K^+ and Cl^- during RVD (Hoffmann et al., 2009). This may occur by electroneutral ion transport pathways, or by the separate activation of K^+ and Cl^- channels. Loss of organic anions and osmolytes also may occur during RVD.

Species	Ion channels	Configu-ration	Unitary conductance (pS)	selectivity	inhibitors	modulators	references
Lamprey *(Lampetra fluviatilis)*	K^+	CA, IO, WC	25	K^+>>Na^+	Ba^{2+}	swelling	(Virkki & Nikinmaa, 1996; Virkki & Nikinmaa, 1998)
	K^+		65	K^+>>Na^+		Mg^{2+} block	
Lamprey *(Petromyzon marinus)*	K^+	CA, IO, WC	80 inward 35 outward	K^+ (1) >Rb^+(2.0) >Cs^+ (4.6) >Li^+ (17.2) >Na^+ (22.4)	Ba^{2+}, ATP, glibenclamide, lidocaine		(Lapaix et al., 2002)
	K^+		25 inward 10 outward	K^+ (1)>Li^+ (2.3)>Rb^+ (2.6) >Cs^+ (6.5) >Na^+ (10.4).	TEA, Ba^{2+}, apamin	swelling	
Dogfish *(Scyliorhinus canicula)*	NSC	CA, IO	18	K^+=Na^+	Ba^{2+}		Unpublished data
Trout *(Oncorhy-nchus mykiss)*	NSC	WC, CA, IO	15	K^+~Na^+~Ca^{2+} >>NMDG	Ba^{2+}, quinine, Gd^{3+}	swelling	(Egee et al., 1997; Egee et al., 2000; Egee et al., 1998)
	SCC		6		NPPB, DIDS glibenclamide		
	ORCC		80		NPPB, DIDS	swelling	
Frog *(Rana sp.)*	K^+	IO	56		ATP, Ba^{2+}	Ca^{2+} increase	(Shindo et al., 2000)
Mudpuppy *(Necturus maculosus)*	K^+	WC			Ba^{2+}	Cell swelling	(Bergeron et al., 1996; Light et al., 2003; Light et al., 2001; Light et al., 1997)
Chicken *(Gallus gallus)*	NSC	WC, IO	24	K^+~Na^+≥Ca^{2+} >>NMDG		stretch	(Lapaix et al., 2008; Thomas et al., 2001)
	NSC		62	K^+~Na^+>>Ca^{2+}>>NMDG		cAMP	
	Cl^-		255			Swelling ?	

Table 2. Ion channels described in vertebrates RBCs.
NSC: Non Selective Cation Channel. SCC: Small Chloride Channel. ORCC: Outwardly Rectified Chloride Channel. CA: Cell-Attached. IO: Inside-Out. WC: Whole-Cell.

A series of articles on *Necturus* erythrocytes showed the common schema of RVD in erythrocytes during hypotonic stress. It was demonstrated that it depends on a quinine-inhibitable K^+ conductance that is regulated during cell swelling by a calmodulin-dependent mechanism (Bergeron et al., 1996), and by a 5-lipoxygenase metabolite of arachidonic acid (Light et al., 1997), as well as by extracellular ATP activation of P2 receptors (Light et al., 2001). Eventually the triggering factor was shown to be the initial increase of intracellular Ca^{2+} concentration (Light et al., 2003). If all other studies do not reach such complete signalling pathway for RVD, it is intriguing and tempting to think that a common schema is conserved throughout evolution of vertebrates RBCs with only minor differences.

Moreover, when RBCs are replaced in their context of respiratory function, and in the light of recent advances on human RBCs regarding anionic transporters, one may think that CO_2/HCO_3^- within the blood occurs originally through anion channels. Indeed, Agnathans (jawless vertebrates) are devoid of Cl^-/HCO_3^- exchangers, but possess like other vertebrates a powerful anion conductance with low selectivity and which presents similar electrophysiological characteristics, as VDAC/PBR found in human RBCs (unpublished data). These types of ionic channel activities have been also reported in trout, chicken as well as in amphibian (table 2). Thus, future comparative studies should go further in deciphering the role played by ion conductance in the success of intracellular O_2/CO_2 transport thought vertebrates evolution. The molecular control in transporters expression and activity and their integrative physiology represent future areas of interest.

6. Conclusion

Though having been explorated by various techniques during decades, the membrane permeability of RBCs is still not fully understood. The use of patch-clamp techniques has proven to be very useful shedding light on a much more complicated situation than expected. Indeed, RBCs are equipped with multiple transporters including various ion channels. These allow precise and fast regulation of volume, acid-base and electrolyte status and they are essential for adequate respiratory functions of RBCs. The external environment of RBCs is constantly changing: with a mean cardiac output of 5L/min and a blood volume of 5L an erythrocyte of healthy human adult has circulate every minute. This means travelling through narrow capillaries (smaller than RBCs own diameter), or bigger vessels in multiple tissues, where acid-base conditions, partial gas pressure, temperature or nutrient concentrations is highly variable. Thus, RBCs are far more than an empty bag of haemoglobin.

There is much evidence that RBCs plays a more complex role than simple oxygen supplier to tissues. Indeed, excessive tissue demand (*i.e.* low oxygen tension) activates RBC signalling pathways resulting in the release of ATP acting in a paracrine fashion to increase vascular calibre (Sprague et al., 2007). RBCs now become vital sensors in matching microvascular oxygen delivery with local tissue oxygen demand (Ellsworth et al., 2009). The pathways involved in this process are not yet fully described, but the presence of calcium pathways plus the Gardos channel and PBR/VDAC provide adequate machinery that could explain these functions of RBCs.

Finally, the characterisation of the various ion channels in diverse vertebrate species is of high interest and constitutes an important field for future research. Indeed if as postulated

above, anion channels originally mediated bicarbonate exchange across RBCs membrane at the root of vertebrates (and Agnathans are a very good model), an evolution process has led to the apparition of Band 3; while anion and cation channels have been maintained in the RBCs membrane throughout this process. Then, the various ion channels in vertebrate RBCs could help describe a phylogeny of respiratory mechanisms throughout evolution, and lead to a better understanding of the role of ion channels in the human RBC membrane.

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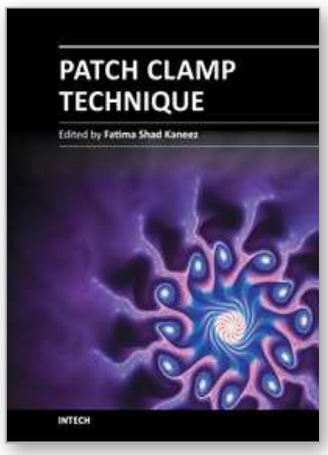

Patch Clamp Technique
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This book is a stimulating and interesting addition to the collected works on Patch clamp technique. Patch Clamping is an electrophysiological technique, which measures the electric current generated by a living cell, due to the movement of ions through the protein channels present in the cell membrane. The technique was developed by two German scientists, Erwin Neher and Bert Sakmann, who received the Nobel Prize in 1991 in Physiology for this innovative work. Patch clamp technique is used for measuring drug effect against a series of diseases and to find out the mechanism of diseases in animals and plants. It is also most useful in finding out the structure function activities of compounds and drugs, and most leading pharmaceutical companies used this technique for their drugs before bringing them for clinical trial. This book deals with the understanding of endogenous mechanisms of cells and their receptors as well as advantages of using this technique. It covers the basic principles and preparation types and also deals with the latest developments in the traditional patch clamp technique. Some chapters in this book take the technique to a next level of modulation and novel approach. This book will be of good value for students of physiology, neuroscience, cell biology and biophysics.

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